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Development of Isolated Mammalian Embryo Techniques for Toxic Substances Screening

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16. ABSTRACT

A potential screen for assessing teratogenic potential of compounds in mammals was tested. The technique involves testing isolated mammalian embryos in culture by direct exposure to agents. Embryos from three species of mammals were used; the mouse, rabbit and swine. Four different substances were studied; 5-fluorouracil, cadmium, cacodylic acid and sodium chloride. About 3300 embryos from mice were used in the development of the technique. In general, the results of the study indicate that young mammalian embryos are responsive to a variety of contaminants that may be found in their environment. Different concentrations of the contaminant will elicit a different kind of response in the embryo. Higher concentrations result in death, but a lower concentration may retard the development of the embryo. Still other concentrations will interfere with the metabolic function of the embryo. Cadmium appears to be the most toxic of the substances studied. It causes the highest rate of embryo death. On the other hand, fluorouracil results in a negative linear relationship between the concentration of the substance and the incorporation of leucine into protein. It appears that some substances are effective over a narrow range while others are effective over a wider range. It is concluded that the procedure as reported is capable of determining the type of effect that a contaminant will have on the development of the mammalian embryo.

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DEVELOPMENT OF ISOLATED MAMMALIAN EMBRYO TECHNIQUES FOR TOXIC SUBSTANCE SCREENING

by

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FOREWORD

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of exisitng and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our Nation's population.

The Health Effects Research Laboratory, Reserach Triangle Park, conducts a coordinated environmental health research program in toxicology, epidemiology, and clinical studies using human volunteer subjects. These studies address problems in air pollution, non-ionizing radiation, environmental carcinogenesis and the toxicology of pesticides as well as other chemical pollutants. The Laboratory participates in the development and revision of air quality criteria documents on pollutants for which national ambient air quality standards exist or are proposed, provides the data for registration of new pesticides or proposed suspension of those already in use, conducts research on hazardous and toxic materials, and is primarily responsible for providing the health basis for non-ionizing radiation standards. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affidavits as well as expert advice to the Administrator to assure the adequacy of health care and surveillance of persons having suffered imminent and substantial endangerment of their health.

The large number of compounds finding their way into the environment has increased the need for reliable short-term testing procedures which will identify those agents in need of further detailed testing. The study described in this report is an effort to develop such a technique for teratology screening using isolated mammalian embryos.

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SCOPE OF WORK

Background and Purpose of Contract. The determination of teratogenic potential of environmental contaminants must be evaluated and become a part of the data base available to the EPA in support of its legislatively mandated regulatory function. Terata are malformations resulting from abnormal growth and development in-utero. Conventional techniques of testing teratological effects of pollutants involve exposure of embryos, in vivo, followed by subsequent analysis of effect on the developed fetus. Such conventional techniques are both time consuming and expensive. There is a need for the development of relatively rapid testing methodologies which will be predictive of, and substitute for conventional teratology testing. Rapid screening methodology is essential if the agency is to address itself to the large numbers of environmental chemical contaminants.

Scope of Work. The contractor shall conduct experiments to develop rapid testing methodologies as substitutes for conventional teratology testing methods. The contractor shall develop teratology screening methods which employ in vitro exposure of embryos to known teratogens for the purpose of determining the feasibility of using embryonic uptake of radiolabeled protein and nucleic acid precursors as biochemical indicators of growth and development.

In the development of screening techniques the contractor shall utilize four compounds for embryo exposures. The compounds shall be subject to approval by the EPA Project Officer and shall include known teratogens, selected pesticides and/or other synthetic organic environmental contaminants. Testing will be conducted on pre-implantation embryos of three mammalian species; mice, rabbits and domestic swine.

The initial testing in development of screening techniques shall be conducted using mice embryos. This testing shall be conducted as follows:

1. Eight-cell stage embryos are to be flushed from the oviduct and grown in an appropriate culture medium that has been proved to support normal growth and development. Experimental embryos will be grown in a medium containing the test substance for up to three days. Their uptake of radiolabeled protein-and nucleic acid-precursors will then be determined as the biochemical indicator of growth and development.

2. Additional exposed embryos will be redeposited into uteri of recipient animals to determine viability following in vitro exposure to these chemicals.

3. Studies shall be performed to assess:

   a. rate of growth during cell divisions occurring in vitro of both exposed and unexposed embryos,

   b. survival rate to term of exposed and unexposed embryos redeposited in uncontaminated uteri of recipient animals.

Upon completion of the above studies involving mice, the contractor shall apply the testing protocols developed to rabbits and domestic swine. The testing of rabbits and swine shall be conducted according to the general methods described above. Upon completion of all testing and studies, the contractor shall provide a Final Report detailing the work performed and recommending specific techniques which may be applied to rapid screening of teratological effects.

REVIEW OF LITERATURE

The thalidomide disaster of 1962 drew the attention of many people to the question of the deleterious effect of drugs and environmental agents upon human reproduction (Epstein, 1973; Nanda, 1975). Prior to this catastrophe little consideration was given to the matter of foreign materials affecting the embryo. For at least the first half of the present century the embryo or fetus was viewed as being protected behind the shield of the placenta and oblivious to the environment of the mother.

Today, the attitude has shifted to one of alarm because of the believed defenseless condition of the developing organism. The actual situation lies between these two extreme views (Wilson, 1975). However, even this actual situation warrants concern since it has been estimated that 2000 new chemicals are developed each year, and that 200 will reach measurable quantities in the environment (Wilson, 1975). Some of these compounds may be carcinogens, mutagens, or teratogens, and the populace does need to be protected from such side effects (Durban and Williams, 1972; Heath et al., 1975). The importance of this is observable in the area of developmental anomalies. Ten to twenty percent of these errors in development can be attributed to gene and chromosomal anomalies. The remainder are believed to occur by interaction of the genotype of a developing organism with its environment (Gottschewski, 1974).

From the standpoint of reproduction, concern about deleterious effects must extend beyond developmental anomalies and include embryo-lethability. It must be noted that these effects are not restricted to just new chemicals, drugs, food additives, pesticides, etc., but that in actuality all compounds at some dosage will be harmful (Sullivan, 1974; Nanda, 1975; Wilson, 1975). The embryo- and fetal-lethality induced by an agent are grouped under the term developmental

toxicity, while a teratogen is a drug, chemical, virus, or physical agent which causes a structural or functional alteration (Staples, 1975). Mechanistically terata are caused by a mutation, a chromosome abberration, an interference with mitosis, an altered nucleic acid synthesis, a lack of precursors for metabolism, an altered energy metabolism, enzyme inhibition, osmolar imbalance, or altered membrane characteristics (Wilson, 1973b).

Adverse effects of these agents can develop at any time during a pregnancy (Nanda, 1975); however, the period of development may be divided into periods of differing susceptibility. It is generally believed that from the time of fertilization until implantation the susceptibility is low, but is present; death of the embryo is the common manifestation of exposure of the embryo to a noxious agent. After implantation and until the completion of organogenesis, the embryo is most vulnerable to assault by some agent. This is the period during which the teratogenic response of the embryo will occur. Finally, from the end of organogenesis until parturition the detrimental effect of a compound will be exhibited as reduced growth or a functional deficiency (Sullivan, 1974; Nanda, 1975; Wilson, 1975; Wilson, 1973a).

The task of evaluating compounds for deleterious effects has been a difficult one. Suggestions for a protocol for drug evaluation have ranged from the very simple to the very complex. A simplistic design is the one given by Sullivan (1974) based upon the sensitivity of the mother vs the fetus. If the dose required to induce abnormalities in the fetus approaches that dose which is toxic to the mother, then the drug or chemical, etc., should be relatively safe. When anomalies are induced at levels well below those of maternal toxicity the compound is unsafe. Examining known teratogen in this light has revealed that most would have been identified as safe compounds and would have passed this test.

Following the thalidomide disaster, the Food and Drug Administration initiated the three generation reproduction tests for pesticides and drugs. These regulations were eventually extended to cover tests for teratogenicity (Epstein, 1973).

These regulations were designed to test the low dosage, long term effects of pesticides and food additives, and later extended to other environmental agents. For the purpose stated, these guidelines are adequate, but for studying the teratogenicity of these compounds, the procedures will not suffice because some health problems arise from short term exposure if it occurs at a critical time. Suggested protocols have been prepared which view the question of short term effects. The FDA evaluation is divided into three phases: 1) fertility and general reproduction, 2) teratology study, 3) prenatal and postnatal effects. In phase I males are treated before mating and females treated before mating, during pregnancy, and during lactation. The young are examined at 13 days gestation, at term, and during nursing. During phase II, treatment occurs on days 6 through 15 and the fetuses are examined 1 to 2 days before term. The prenatal and postnatal phase is composed of treatment during the last third of pregnancy and throughout lactation. The offspring are evaluated for survival and growth (Wilson, 1975). The common test species are laboratory animals (rats, mice, hamsters, rabbits, etc.).

The evaluation of drugs for side effects encounters a number of problems with these recommendations. Of greatest difficulty is the problem of metabolism. Many compounds as they exist in the environment do not have adverse effects, but occasionally from the metabolites found by an organism there are teratogenic, carcinogenic, or mutagenic effects (Druckrey, 1974). Therefore, it is important that the species used for testing metabolizes the compound in a manner similar

to that in the human. If this is not the case a harmful compound may pass the examination or a safe substance may be blocked from distribution. The metabolic similarity of the test species must be examined from the standpoint of the embryo, the dam, and the placenta. Not only is the placenta important because of its ability to metabolize compounds but also because of its transport function. By regulating the passage of many materials to the embryo it regulates the embryo's exposure to environmental agents. This variable of exposure must be considered in screening methodologies as part of dosage. The transport functions within the mother will alter the level of the agent reaching the embryo. These considerations must be under advisement when a dosage for an experimental animal is selected; in addition, the route of administration must be as close to that of the human as possible (a dietary component being investigated should not be administered intravenously). Examination of the test animals should be thorough; not superficial.

If the compound is tested for teratogenicity the fetuses should be examined both internally and externally (Palmer, 1974; Wilson, 1975; Lister, 1974). Within such screening studies the number of animals is often small. Ideally the number should be large. Especially since a small increase in occurrence of a rare deformity is significant; it may require hundreds or thousands of litters to detect just a dabbling in the rate of such an occurrence (Palmer, 1974; Wilson, 1975; Epstein, 1973).

To alleviate these difficulties Wilson (1975) has proposed the following changes for improving teratological listing:

1) Use short duration dosage as well as long term.
2) Use one species other than rabbits and rodents which preferably metabolizes as humans do.

3) Determine embryotoxicity levels for experimental animals and project downward to an acceptable level for man; not up from an estimated maximum dose.

4) Evaluation of postnatal function

5) Use primates to screen agents likely to be used during human pregnancy.

In 1969 an advisory panel for the Department of Health, Education and Welfare recommended that:

1) Teratogenic effects should be studied in experimental animals.

2) Test agents should be administered during active organogenesis.

3) Parameters should be a) the incidence of abnormal litters, b) the number of abnormalities/litters and c) the incidence of specific congenital abnormalities, d) the incidence of fetal mortality, e) maternal weight gain during pregnancy and f) maternal and fetal organ/body weight ratios.

4) Some animals should be allowed to give birth to determine abnormalities in the prenatal period.

5) Two species of animals should be used.

6) The animals should be of various nutritional conditions.

7) A variety of routes of administrations should be used.

8) Enough dosages should be used to cover high accidental exposure and low chronic exposures (Epstein 1973).

These HEW proposals and those of Wilson (1975) would complicate the present screening procedures and greatly increase the cost. To circumvent this Wilson (1975) calls for a shortcut method of screening. This may be advised by understanding the mechanism of teratogenicity and relating new compounds to these mechanisms. Also, simple biological systems may provide information about the deleterious effect of the compound being screened. Similarly, Axelrod (1972) has called for a system that would be rapid and objective. He suggests that this might

be achieved through small animal models and/or biochemical test which would provide a data on changes that occur before anatomical malformations would be observable. He suggests the use of enzyme action as an end point.

A number of possibilities of rapid and thorough screening systems have been investigated. These have ranged from the culture of bacteria to the in vitro growth of post implantation mammalian embryos. Some degree of industrial acceptance has been given to a "quick" test for evaluating new compounds for carcinogenicity. On the belief that many carcinogens are mutagens a test was developed by Bruce Ames which consists of observing strains of Salmonella (after exposure to a chemical) for mutations from a histidine dependent strain to one that is not dependent upon histidine's being present in the culture media. Though a number of industries have accepted this test the National Cancer Institute has not endorsed it (Kalato, 1976).

Considerable effort was expended upon the development of a teratogen screening system utilizing chick embryos. However, acceptance did not occur since it appeared as though these embryos were too susceptible to chemically induced malformations. Also, little success was achieved with compounds other than those that were water soluble. Results of this system encouraged some to propose further evaluations (Gebhardt, 1972), while others saw the results as a menace to human safety (Goldberg, 1975).

For screening systems the utilization of various types of cell and tissue culture techniques have been prepared by Moscona (1975). These consisted of studying the effect of agents upon 1) cleavage of the egg, 2) growth of embryonic myocardial and skeletal muscle cells in culture, 3) innervation of muscle in vitro, 4) reconstruction of tissues from embryonic cell suspensions (e.g. chondrocytes to cartilage), 5) interactions between epithelial and mesenchymal tissues, and 6) induction of systems by hormones. In vivo, alterations in each of these systems

during embryonic development can result in developmental anomalies. Similarly, Rajan (1974) has proposed the in vitro culture of human organs as test tissue until it is possible to grow undifferentiated human embryos in vitro.

Organ culture of fetal tail vertebrae has been utilized to show a detrimental effect of 5-fluorouracil (Yashihara, 1968). This pyrimidine is a patent teratogen, and in this culture system showed a detrimental effect upon the development of the vertebrae with a definite relationship between the length of exposure and the degree of deformity.

The value of organ culture in testing for a teratogen has been demonstrated by Kochbar (1975) with the culture of mouse limb buds. Detrimental effects of vitamin A, bramadeoxyuridine, antiglutaneres, thalidomide, B-ameropropicnitribe have occurred when limb buds have been exposed in vitro. It has been found that organs cultured in this manner are more sensitive to teratogenic agents than when in the embryo; however, with further testing such a system may provide a reliable indicator of teratogenicity.

There has been developed by D.A.T. New (1975) a system for culturing post implantation embryos of rodents. Rat embryos of 7 to 15 somites can be cultured relatively successfully to 28 somites, and 22 to 28 somites can be cultured to 40 somites. Possible usage for teratogen screening has been reported by New (1975) and the following advantages of the system listed: a) low cost and simple culture media required of rodents b) embryos grown successfully during the period of organogenesis c) direct observation of the embryos are allowed d) precise control of test conditions e) unsuspected causes of malformation revealed and f) freedom from maternal metabolism and possibly of placental metabolism. The last can also be a disadvantage along with growth periods of only 1 to 3 days.

In 1966 Turbow reports using the above system to evaluate the teratogenic dye

trypan blue. Low and high concentrations of the dyes were tested. Teratogenic effects were observed at low concentrations when the embryos were injected within the yolk but not when the embryos with an intact yolk sac were immersed in the dye. At high concentrations a deleterious effect occurred with either method.

Modification of New's procedure has been reported by Kochbar (1975) and used to test known teratogens by measuring the incorporation of ^{3}H-thymidine into DNA and labelled praline into proteins. Both vitamin A and DON (6-diazo-5-oxanorleucine) caused a reduction in the incorporation of ^{3}H-thymidine into DNA. The degree of depression was positively related to the level of teratogen added to the culture medium.

Vitamin A also reduced the level of protein synthesis. DON was not reported as being part of the amino acid study. This discussion of methodology has presented a variety of techniques which have been evaluated as possible tests for deleterious effects of environmental agents. One area that has not been fully investigated for contributions to this field is the use of preimplantation mammalian embryos.

One area of use of preimplantation embryo methodologies has been the attempt to separate the susceptibility of the embryo to a teratogen from effects upon the embryo as a result of a maternal response to the teratogen. The procedure used was the transfer of embryos prior to implantation from one genetic strain of mice to another (one strain of high susceptibility and one of low susceptibility). In two cases in which glucocorticoids were administered during organogenesis to susceptible strains of mouse embryos (Ajax and Swiss) after they had been transferred to resistant strains, the number of anomalies was reduced (Vetter, 1971; Takano, et al., 1972).

Using the same procedure Marsk et al. (1971) found the susceptibility to be

embryonic in nature since the number of anomalies was not reduced when embryos previously transferred to a resistant strain were treated with cortisone. The same procedure was used by Marsk et al (1975) to study sodium salicylate. In this case the embryos of a resistant strain transferred to a susceptible strain increased in number the terata observed.

Embryo transfer has shown that some compounds do have an effect during the preimplantation period that will cause developmental anomalies. Treatment of mice with cyclophosphamide prior to transfer decreased the number of embryos surviving after transfer as compared to controls and a malformation of the maxilla occurred in transferred embryos which did not occur in treated untransferred controls. With actinomycin D in treated untransferred controls the number of embryos surviving decreased with increasing dosages of the drug. There was no observable effect on survival rate and no malformations in treated transferred embryos (Speilmann et al., 1974).

R. L. Brinster (1975) has discussed from a broad viewpoint the possible roles which preimplantation embryos could play in teratology. He believes that they could be used to investigate the mechanism of action of teratogens upon embryonic cells. Normally the only effect of exposure of preimplantation embryos to teratogens is death; the response may result because of the small number of cells composing the embryo at such stages. In these embryos which die the mechanisms of cellular alteration may be the same as those which occur during abnormal organogenesis. During the organogenesis surviving cells are large enough in number to form the organ, but in an anomalous form.

Brinster proposed that parameters for a screening system could be either morphology of the embryo or measurements of metabolic capabilities.

The morphological aspect has been investigated. In 1964, M.F. Hay reported

an altered morphology of rabbit blastocyst after the dam had been treated with thalidomide. Lutwak-Mann et al. (1969) examined the effects of anti-metabolites, metabolic inhibitants, anti-mitotic agents, metabolites, and 0_2 on rabbit blastocysts exposed in vivo and cultured in vitro; cultured and exposed in vitro, and exposed in vivo and not cultured.

From the study no general conclusion about a group of embryos could be drawn. Many compounds had no effect when the dams were treated and the embryos cultured in vitro, but did have an effect when exposure was in vitro. Only 6-mercaptopurine riboside had an effect when treatment was given the dam, and no effect on blastocyst morphology when exposure was in vitro. All other in vivo effects occurred in vitro. When embryos were exposed in vivo and cultured in vitro the most common response was no change in morphology during culture. An exception was 2-deoxyglucose which had a detrimental effect in vivo, but the embryos recovered in vitro.

In their discussion the authors warned that in vivo testing would always be needed, and that some compounds showed no effect on the blastocyst in vivo or in vitro but are known to be teratogenic when given during organogenesis.

In other sections of this review it was noted that several authors have called for a biochemical analysis which could be used to test for deleterious effects of environmental agents. Brinster (1975) proposed the measurement of metabolic functions of preimplantation embryos as such a system. Such a proposal can be made because a large volume of literature exists on the metabolism of the preimplantation embryo. Nucleic acid synthesis in the mouse has been investigated with the use of auto-radiography and found to occur at a much earlier stage of development than in amphibians (Izquierdo, 1965); duration of phases of the cell cycle has been determined by Luthardt and Donahue (1975) using this technique. Qualitative and quantitative measurements of nucleic acid synthesis have been made using labelled

precursors and techniques of separating various classes of nucleic acids in the mouse (Monesi and Salfi, 1967; Ellem and Gwatken, 1968; Piko, 1970; Monesi et al, 1970; Epstein and Daentl, 1970; Epstein and Daentl, 1971; Daentl and Epstein, 1971; Epstein et al., 1971; and Murdoch and Wales, 1973) and in the rabbit (Manes, 1969; and Manes, 1971). Protein synthesis has been monitored in the mouse by determining the amount of radioactivity incorporated into protein from labelled amino acids (Monesi and Salfi, 1967; Thomson and Biggers, 1966; Brinster, 1971; and Epstein and Smith, 1973) and also in the rabbit by the same methodology (Manes and Daniel, 1969). Proteins synthesized during various cleavage stages of mouse (Van Blerkom and Brockway, 1975) and rabbit (Van Blerkom and Manes, 1974) have been separated by polyacrylamide gel electrophoresis and quantitatively evaluated using autoradiography. The sensitivity of such procedures for detecting variations in metabolism have been demonstrated with investigations upon altered embryo development. Using autoradiography, Sanyal and Meyer (1970 and 1972) observed that delayed implantation of rat blastocysts resulted in fewer nuclei incorporating ^{3}H-thymidine than in controls and that delayed embryos treated with estrogen and progesterone to simulate the conditions at implantation had an increased level of incorporation as compared to untreated delayed embryos. A similar observation was made with ^{3}H-uridine (Jacobson et al., 1970).

Investigating mouse embryos undergoing delayed implantation as a result of ovarectomy, Weitlauf (1972) found that these blastocysts had a lower level of incorporation of labelled amino acids into protein than did controls. It was also observed that after culturing delayed blastocysts for 8 hours, the incorporation increased to near that of controls. This observation is an example of the delineation of a maternal inhibitory effect utilizing biochemical techniques on preimplantation embryos.

Direct effects of hormonal steroids upon embryos have been investigated in rabbits and mice. Daniel (1964) observed fragmentation of rabbit embryos cultured *in vitro* with estrogen and cleavage inhibition with progesterone. It was later found that the cleavage inhibition was reversible if the embryos were placed in fresh media or if the level of proteins and amino acids in the culture was increased (Daniel and Levy, 1964). Synthetic steroids of oral contraceptives were also observed to cause inhibition and fragmentation of preimplantation rabbit embryos (Daniel and Cowar, 1966). The incorporation of ^{14}-C labelled amino acids into mouse embryos was found to be unaffected at the morula stage, slightly increased in early blastocyst, and significantly increased in expanded blastocysts following growth in culture media containing estradiol 17-B (Smith and Smith, 1971). An example of the valuable information about environmentally induced problems that can be obtained from investigations with preimplantation embryos has been reviewed by Ulberg and Sheean (1973). Periods of transitory loss of fertility occur in many species following exposure of the female to high environmental temperatures. In cattle and sheep a decrease in fertility occurs for each degree rise in body temperature during the time of the first cleavage. Four and eight cell rabbit embryos transferred from heat stressed females to normal does failed to survive. No effect was observed when unfertilized ova from control and heat stressed ewes were transferred to ewes whose reproductive tract contained spermatozoa; this observation demonstrated that the detrimental effect of increased body temperature occurs after fertilization. Heat stressing of spermatozoa *in vivo* reduced embryo survival rate; exposure of first cleavage rabbit embryos *in vitro* resulted in decreased survival and retarded development. Mouse embryos respond in the same manner as other species mentioned. *In vitro* culture of heat stressed embryos with ^{3}H-uridine indicated an increased incorporation into RNA above that of control embryos.

With this example it is possible to see the identification of an environmental problem (reduced fertility); the delineation of the level of the problem (the embryo); and the biochemical evaluation of the effect of the environment (increased RNA synthesis).

With results such as these, and those on delayed implantation, and hormonal effects the utilization of these methodologies for possible screening of effects of environmental agents is certainly warranted.

The purpose of the investigation being reported was to evaluate the use of preimplantation embryo culture as a screening system for the deleterious effects of environmental agents. The parameters used for evaluation of the system were the ability of the embryos to cleave, the rate at which development occurred, the survival of embryos after transfer to dams, and incorporation of ^{3}H-leucine into proteins.

MATERIALS AND METHODS

Animal Strains. Mice were of an Institute of Cancer Research randombred stock maintained in the Animal Science Department, North Carolina State University. Rabbits were New Zealand whites obtained from the Franklin Rabbit Ranch, Wake Forest, North Carolina. Virgin females were of an 8-1/2 lb. minimum weight. Pigs were crossbred gilts and boars from an experiment station research farm. One boar was a pure bred Yorkshire.

Culture Media. For constituents of the culture media see the appendix. Mouse and rabbit embryos were grown in Brinster's Medium for Ova Culture II ($BMOC_2$); pig embryos were cultured in synthetic oviduct fluid (SOF).

Embryo Recovery and Culture

Mouse Embryos. Normally ovulating female mice were sacrificed on day 2 (day 0 = day of vaginal plug) by cervical dislocation. The oviducts were excised, and flushed with $BMOC_2$ utilizing a 30 gauge needle, 2 cc syringe and a Wild binocular dissecting scope with 12x magnification. Embryos were pooled from all females on a given day, washed once, separated into groups of 10 eight-cell embryos (at 50x) and placed in appropriate 10 x 75 mm culture tubes containing 1 ml of $BMOC_2$.

Rabbit Embryos. Superovulated rabbits (see appendix for regime) were sacrificed by exsanguination and embryos recovered from the oviduct by flushing it with 5 ml of $BMOC_2$ into a petri dish approximately 42 hours after mating. Eight-cell embryos were pooled, washed once, separated into groups of 5, and placed in appropriate tissue culture wells containing 1 ml of $BMOC_2$. Eight culture wells were located in a covered petri dish containing approximately 20 ml of water.

Swine Embryos. Pig embryos were flushed from the uterus and oviduct with SOF for flushing (see appendix) on either day 3 or 4 (day 0 = 1st day of estrus) under surgical conditions. Embryos were pooled, washed once, grouped according to cell

stage, and divided evenly (according to cell stage) between control and treatment groups and placed in 10 x 75 mm culture tubes containing 1 ml of either treated or untreated SOF.

Incubation. The 10 x 75 mm culture tubes and petri dishes containing the culture wells were placed in an incubator at 37°C with an atmosphere of 5% CO_2 and 95% air prior to the addition of embryos. Humidity was maintained with an open container of water.

Experimental

The culture media into which embryos were placed contained either no environmental agent or one of four compounds being investigated. The compounds and their chemical form were: cadmium ($CdCl_2$), 5-fluorouracil, cacodylic acid (sodium cacodylate) and NaCl. The concentrations of the materials used were calculated upon the basis of the moeity of interest, and the appropriate amount was added to the culture medium. On a given day the embryos were divided between at least one control culture and several dilutions of one contaminant.

Mouse Embryos. Following 24 hrs of culture 5 μCi of ^{3}H-leucine (specific activity 60 Ci/mmole, New England Nuclear) in 50 μl of H_2O was added to the control, each of the dilutions of the contaminant, and at least one tube containing 1 ml of $BMOC_2$ but no embryos (blank for incorporation measurement). After 3 additional hours of culture the embryos were removed from an individual culture tube, placed on a watch glass and classified at 50x as cleaved (ICM-expanded blastocyst with inner cell mass), blastocyst (small blastocoele cavity), morula, or abnormal (16 or 32 cells, or irregular morphological conformation), not cleaved, degenerated (blastomeres lyzed and cytoplasm contained within the zona pellucida) or fragmented (repeated cytoplasmic division of the cytoplasm, but no nuclear division of the blastomeres). Cleaving and non-cleaving embryos were separated and were

transferred to 1 ml of cold $BMOC_2$ in separate 10 x 75 mm culture tubes.

The contents were decanted onto a Millipore filter (.45μ pore size) and filtered with a vacuum. The tube was rinsed three times with 1 ml of cold 5% TCA (trichloroacetic acid) and a total of 25 ml of acid was passed through the filter. The filters were placed in a scintillation vial, dried, dissolved in a scintillation cocktail, and radioactivity determined in a Packard Tri-Carb liquid scintillation spectrometer. By a computer transformation the counts/minute were converted to disintegrations/minute using data from an automatic external standard.

Rabbit Embryos. Following 24 hours of culture 25 μCi of ^{3}H-leucine (specific activity = 53 Ci/m mole, Amersham-Searle) in 50 μl of H_2O was added to the control and each of the dilutions of the contaminant. After 3 additional hours of culture the embryos were classified at 50X in their culture wells as cleaved (16 cell or morula), not cleaved, degeneratred, or fragmented. Embryos were transferred to a 10 x 75 mm culture tube containing 1 ml of $BMOC_2$ + leucine (0.014g./liter). A volume of $BMOC_2$ equivalent to that used to transfer the embryos was placed in a separate tube and processed as a blank. The solution was frozen at -20°C and stored at least overnight. Following rapid thawing the embryos were rapidly frozen (dry ice and acetone or liquid nitrogen) and thawed at 37°C five times; 2 ml of 10% TCA was added to each tube, and the tubes stored at 4°C overnight. Contents were decanted onto a Millipore filter (.45μ pore dize) and filtered. Two ml of an ice cold 10% TCA washing were passed through the filter followed by 10 ml of hot 10% TCA (80-90°C) and 5 ml of ice cold 70% ethanol. The filters were placed in scintillation vials, dried, dissolved in scintillation fluid and counted as were the mouse embryos.

Swine Embryos. After equal division of the embryos according to cell stage, 5 μCi of ^{3}H-leucine (specific activity 60 Ci/m mole, New England Nuclear) in 50 μl

of H_2O was added to each culture tube. Following 24 hours of culture the embryos were removed from their culture tubes, placed on a watch glass, and classified as to stage of cell division. The embryos were then placed in 1 ml of cold $BMOC_2$ and decanted onto a Millipore filter and treated in the same manner as were the mouse embryos.

Transfer of Cultured Embryos to Pseudopregnant Recipients. Embryos were grown in vitro as described previously. At the end of 24 hours the embryos were classified, and picked up with a finely drawn glass pipette. The uterus (exposed through mid-ventral laparatomy-rabbits; high lumbar laparatomy-mice) was punctured with a hypodermic needle (mice - 27 gauge; rabbits - 20 gauge); the pipette was inserted; and the embryos were expelled into a uterine lumen.

All females were pseudopregnant. Mice were day 2 (day 0 = vaginal plug from vasectomized male) and rabbits were approximately 66 hours pseudopregnant (induced by mating and removing embryos at 42 hours, or by LH injections).

Photography. All photographs of embryos were taken as wet mounts with a camera attached to a binocular microscope.

Statistical Evaluation. Cleavage patterns were analyzed by chi-square analysis; leucine incorporation by control and treated embryos was evaluated by least squares analysis of variance; and treated embryos were examined for a linear relationship between the level of contaminates and leucine incorporation by regression analysis.

RESULTS

Mouse Embryos

Over 3300 8-celled embryos were used to investigate the effect of cadmium, 5-fluorouracil, cacodylic acid, and sodium chloride on the development of preimplantation embryos. The results of these experiments are presented in Tables 1-7, Figures 1-7, and Photographs 1-8.

Cadmium. A dramatic effect of cadmium in embryo development is presented in Table 1 and Figure 1. The percentage of embryos cleaving is significantly reduced after treatment with 1 ppm or 100 ppb Cd (0 and 12%, respectively) as compared to controls (99%). Cadmium treated embryos were not classified as to stage of development. At 100 ppb Cd the level of incorporation of ^{3}H-leucine was significantly reduced to only 13% of the control value, while at 10 ppb the incorporation was significantly elevated above controls (2486 DPM vs 1592 DPM). Embryos characteristic of controls and Cd treatment (1 ppm) are presented in Photographs 1 and 2, respectively.

5-Fluorouracil. Table 2 and Figures 2 and 3 clearly illustrate the deleterious effect of 5-fluorouracil (F) on embryo development. The number of embryos cleaving is significantly reduced at 100 ppm, 10 ppm and 1 ppm F (76%, 86%, 93%) vs controls (100%).

The embryos treated with fluorouracil were classified as to stage of development at the end of the culture period. There is a dramatic retardation of development at 100 ppm, 10 ppm, and 1 ppm F. Besides growth retardation there is also an obvious morphological abnormality in these embryos. The frequency of development of this malformation decreases with increasing dilution of the antimetabolite. Embryos treated with fluorouracil are presented in Photographs 3, 4, 5, 6 and 7. Leucine incorporation was significantly reduced by all of the fluorouracil dilutions (Table 3). There exists a highly significant ($P < .001$) linear relationship between the level of fluorouracil treatment and the incorporation of leucine. For this analysis by linear regression the control data were excluded.

PHOTOGRAPHS

1. Untreated (control) embryos - morula stage

2. Cadmium treated embryo - uncleaved degenerating embryo characteristic of treatment with 1 ppm and 100 ppb Cd

3. & 4. Fluorouracil treated embryos (100 ppm) - embryos show abnormal cleavage characteristic of fluorouracil treatment*

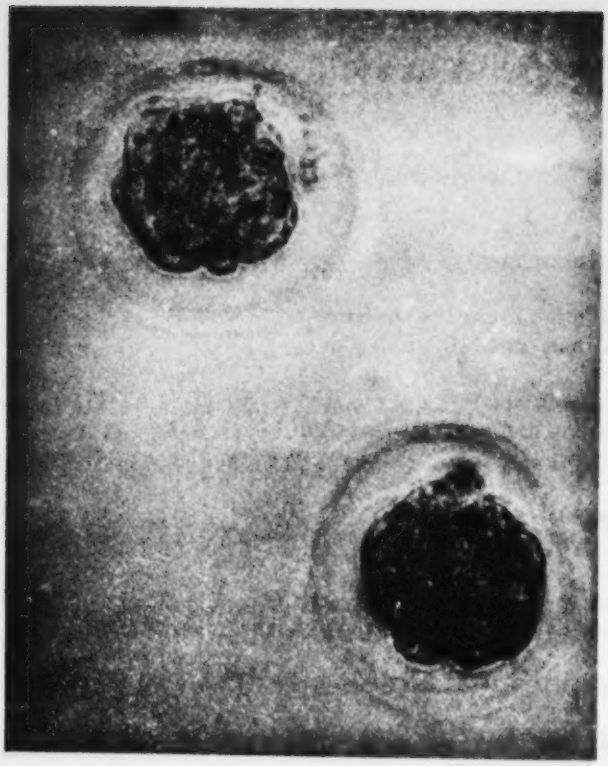
5. Fluorouracil treated embryos (10 ppm) - 3 abnormally cleaved embryos and 1 resembling a normal embryo*

6. Fluorouracil treated embryos (1 ppm) - 3 normal appearing embryos and one abnormal*

7. Fluorouracil treated embryos (100 ppb) - 2 normal morula*

8. Uncleaved 8-cell embryo representative of treatment with 10 ppt or 1 ppt cacodylic acid or 10 ppt NaCl

*Ratio of normal and abnormal is characteristic of the treatment group.



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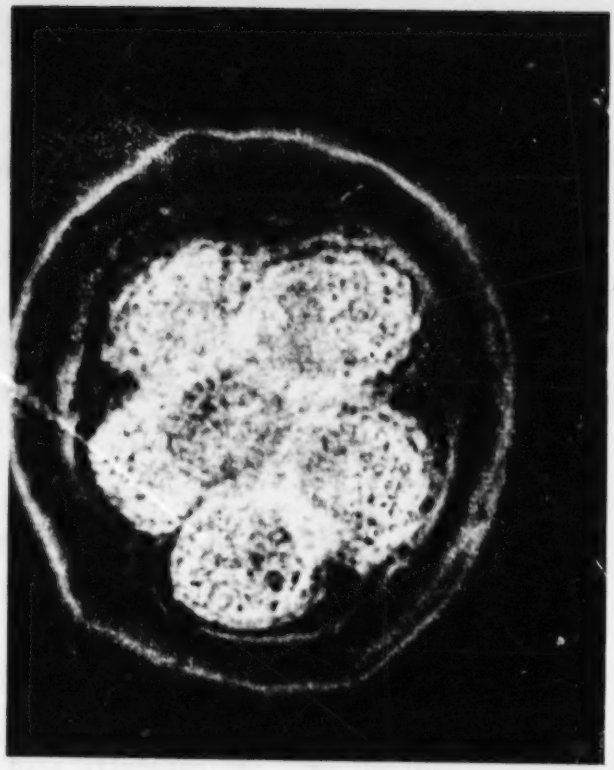

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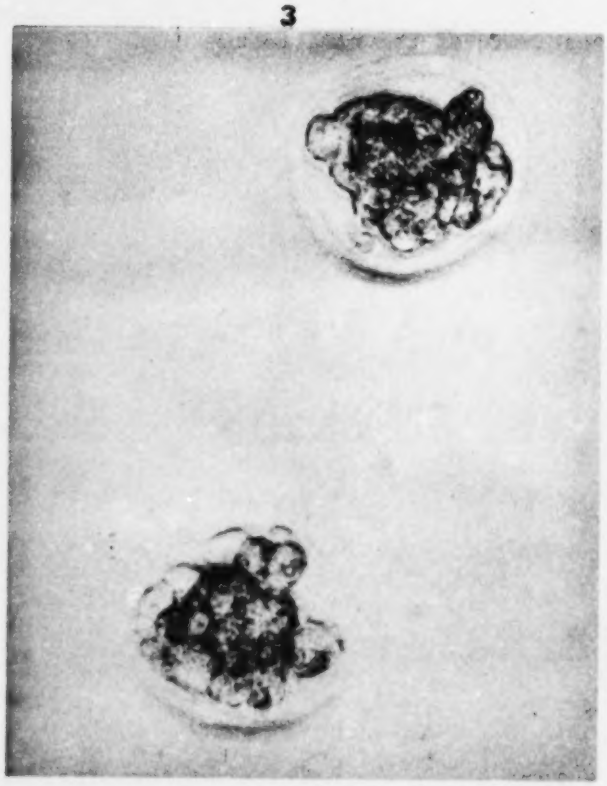

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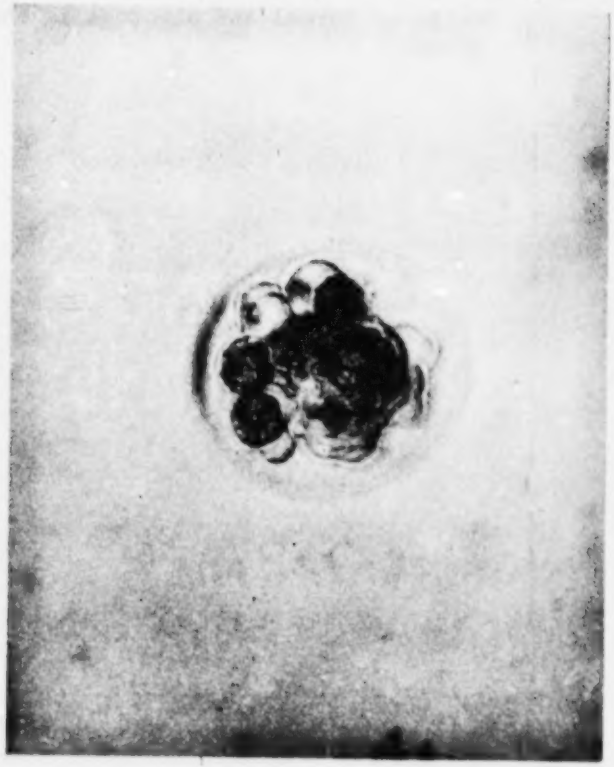

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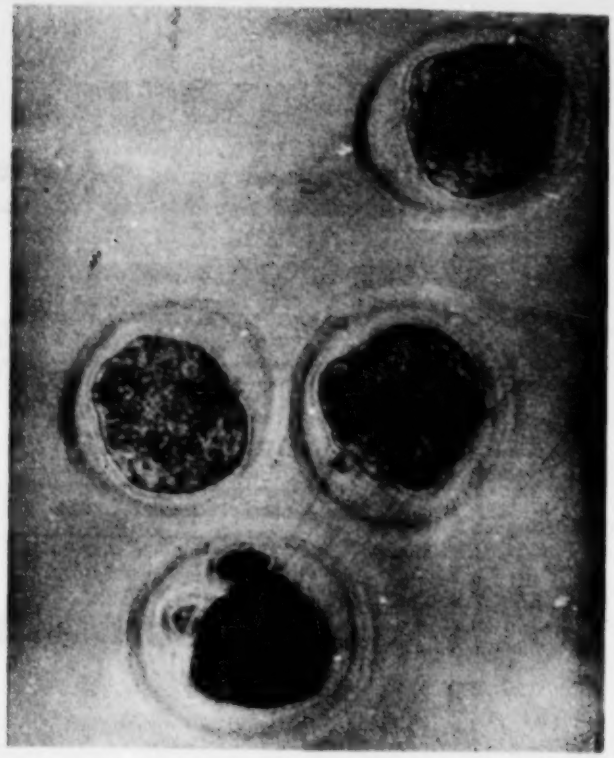

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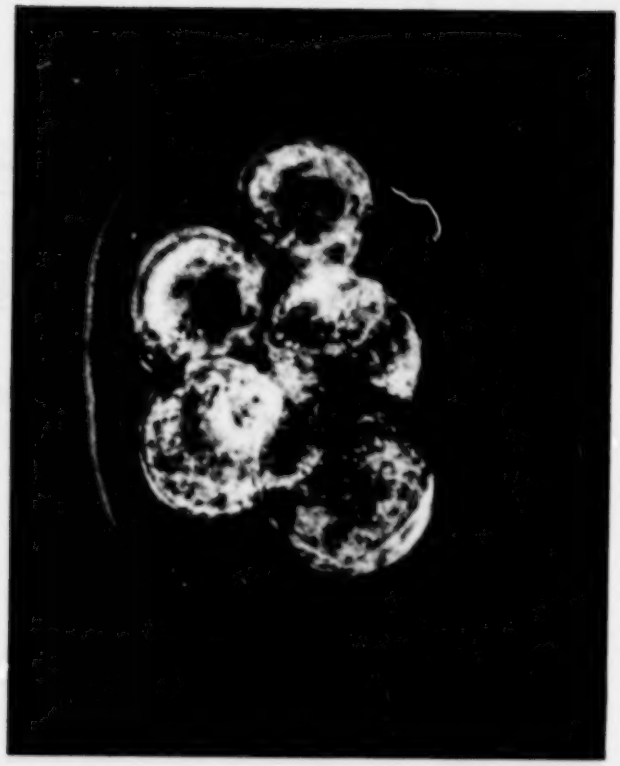

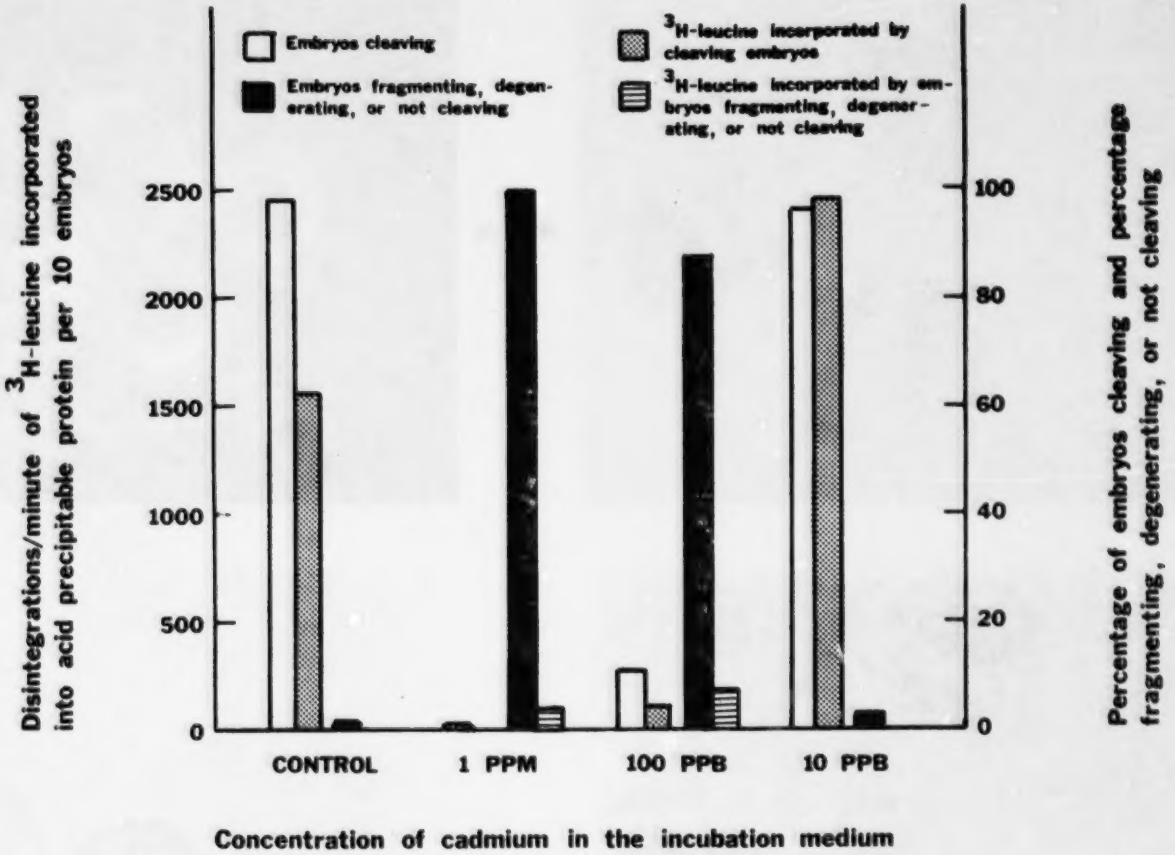

Fig. 1 Incorporation by mouse embryos of ^{3}H-leucine into acid precipitable protein after 24 hours of incubation with cadmium

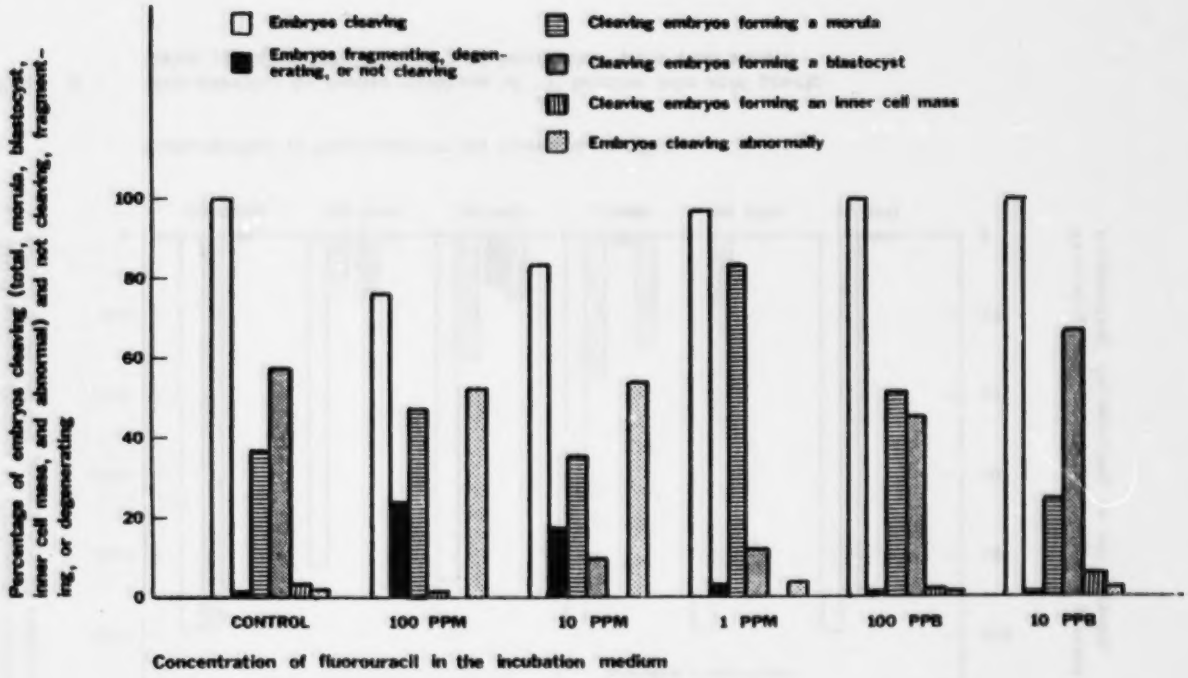

Fig. 2 Developmental response of mouse embryos following 27 hours of culture with fluorouracil

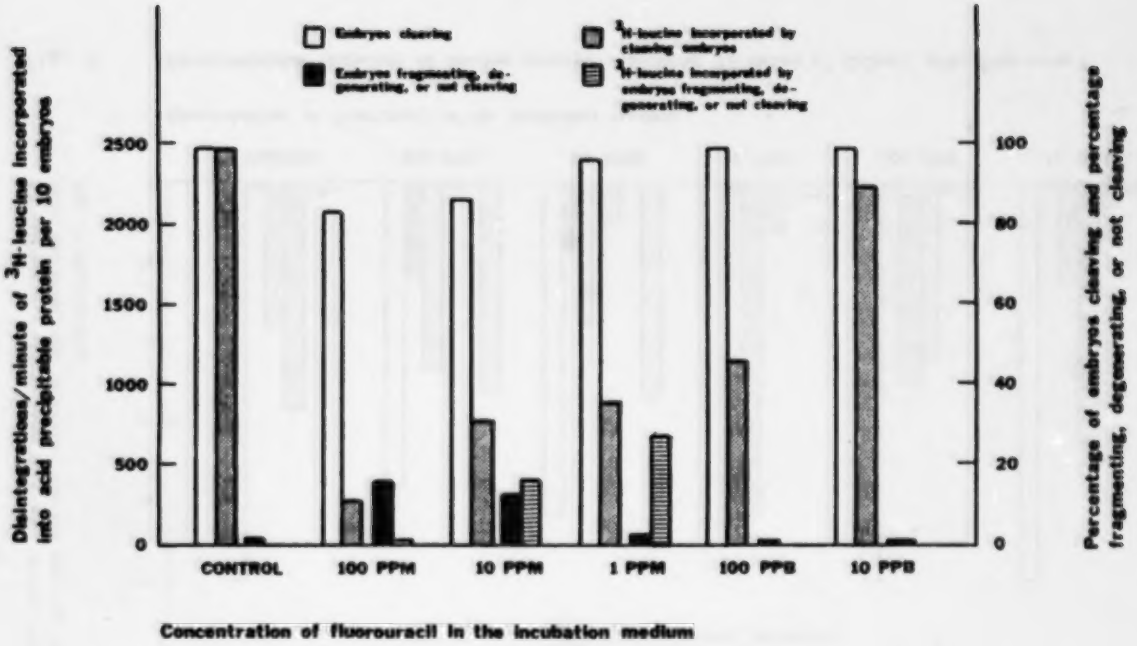

Fig. 3 Incorporation by mouse embryos of ^{3}H-leucine into acid precipitable protein after 24 hours of incubation with fluorouracil

Table 1. Developmental and metabolic response of mouse embryos cultured in the presence of cadmium

Treatment	Total No. of embryos	Embryos cleaving				Embryos fragmenting, degenerating or not cleaving			
		No. of incubations	No. of embryos	Percent of total	DPM	No. of incubations	No. of embryos	Percent of total	DPM
Control	277	29	274	99	1592	3/0[a]	3/0[b]	1	---
1 ppm	220	---	---	0[c]	----	24	200	100[c]	104
100 ppb	219	3	27	12[c]	139[c]	21/20[a]	192/191[b]	88[c]	247
10 ppb	280	31	273	98	2486[c]	6/0[a]	7/0[b]	2	---

[a]Number of incubations in which embryos responded in a given manner/number of these incubations used for incorporation determinations

[b]Number of embryos having a specific response/number of these embryos used for incorporation determinations

[c]Significantly different from control (P .05)

Table 2. Developmental response of mouse embryos cultured in the presence of fluorouracil

Treatment	No. of incubations	Total No. of embryos	Embryos fragmenting, degenerating, or not cleaving	Embryos cleaving	Stage of development of cleaving embryos			
					ICM	B	M	A
Control	13	126	0 0%	126 100%	5 4%	73 58%	48 38%	0 0%
100 ppm	4	38	9 24%[c]	29 76%[c]	0 0%[c]	0 0%[c]	14 48%[c]	15 52%[c]
10 ppm	12	114	19 17%[c]	95 83%[c]	0 0%	10 11%[c]	34 36%[c]	51 54%[c]
1 ppm	11	101	3 3%[c]	98 97%[c]	0 0%[c]	12 12%[c]	82 84%[c]	4 4%[c]
100 ppb	9	85	0 0%	85 100%	2 2%	39 46%	44 52%	0 0%
10 ppb	7	67	0	67 100%	4 6%	45 67%	17 25%	1 2%

[c]Significantly different from control ($P < .05$)

Table 3. Metabolic response of mouse embryos cultured in the presence of fluorouracil

Treatment	Total No. of embryos	Embryos cleaving				Embryos fragmenting, degenerating or not cleaving			
		No. of incubations	No. of embryos	Percent of total	DPM	No. of incubations	No. of embryos	Percent of total	DPM
Control	126	13	126	100	2522	---	---	0	---
100 ppm	56	6	47	84	293[cd]	3	9	16	0
10 ppm	124	13	105	85	796[cd]	5/2[a]	19/13[b]	15	429
1 ppm	101	11	98	97	916[cd]	3/0[a]	3/0[b]	3	---
100 ppb	85	9	85	100	1263[cd]	---	---	0	---
10 ppb	75	8	75	100	2249[cd]	---	---	0	---

[a]Number of incubations in which embryos responded in a given manner/number of these incubations used for incorporation determinations.

[b]Number of embryos having a specific response/number of these embryos used for incorporation determinations.

[c]Significantly different from control ($P < .05$).

[d]Significant linear relationship between the concentration of fluorouracil and incorporation of leucine ($P < .001$). Does not include control data.

Cacodylic Acid. Cacodylic acid had a much less dramatic effect upon embryo development and metabolism than did the previous compounds. Concentrations of 10 ppt and 1 ppt were required to inhibit cleavage, and the only treatment which resulted in a significant alteration in development was 1 ppt. Though 87% of the embryos failed to cleave when cultured with 10 ppt cacodylate, the remaing 14% appeared to develop at a normal rate (Table 4, Figure 4). No significant effect of the treatment procedure as a whole was detected; but when the control was omitted and the remaining treatments analyzed for a linear relationship, significance was achieved (Table 5, Figure 5). Photograph 8 is an embryo characteristic of the uncleaved ones resulting from treatment with 10 ppt and 1 ppt cacodylate.

Sodium Chloride. Only treatments of 10 ppt and 1 ppm NaCl significantly prevented cleavage (100% and 14%, respectively) as compared to 7% for control embryos. Treatments with 1 ppt, 100 or 10 ppm resulted in a significant improvement in the ability of the embryos to cleave. A significant retardation in embryo development occurred at 1 ppm, 100 ppb and 10 ppb; a significant improvement took place when the embryos were grown in 10 ppm NaCl (Table 6, Figure 6). Leucine incorporation corresponded to the developmental results. Treatments of 1 ppt, 10 ppm and 1 ppm significantly increased the incorporation of leucine while a significant decrease was noted with levels of 10 ppm, 100, 10 and 1 ppb (Table 7, Figure 7).

Embryo Transfers. Embryos cultured in vitro for 24 hours were transferred to both horns of 75 pseudopregnant females. Of these females 5 were pregnant on day 12. Because of this low success rate transfers of treated embryos were not made.

Rabbit Embryos

Over 800 rabbit embryos were cultured in various concentrations of cadmium, 5-fluorouracil, cacodylic acid, and sodium chloride. During 24 hours of culture in $BMOC_2$ the 8-cell embryo cleaved into a morula.

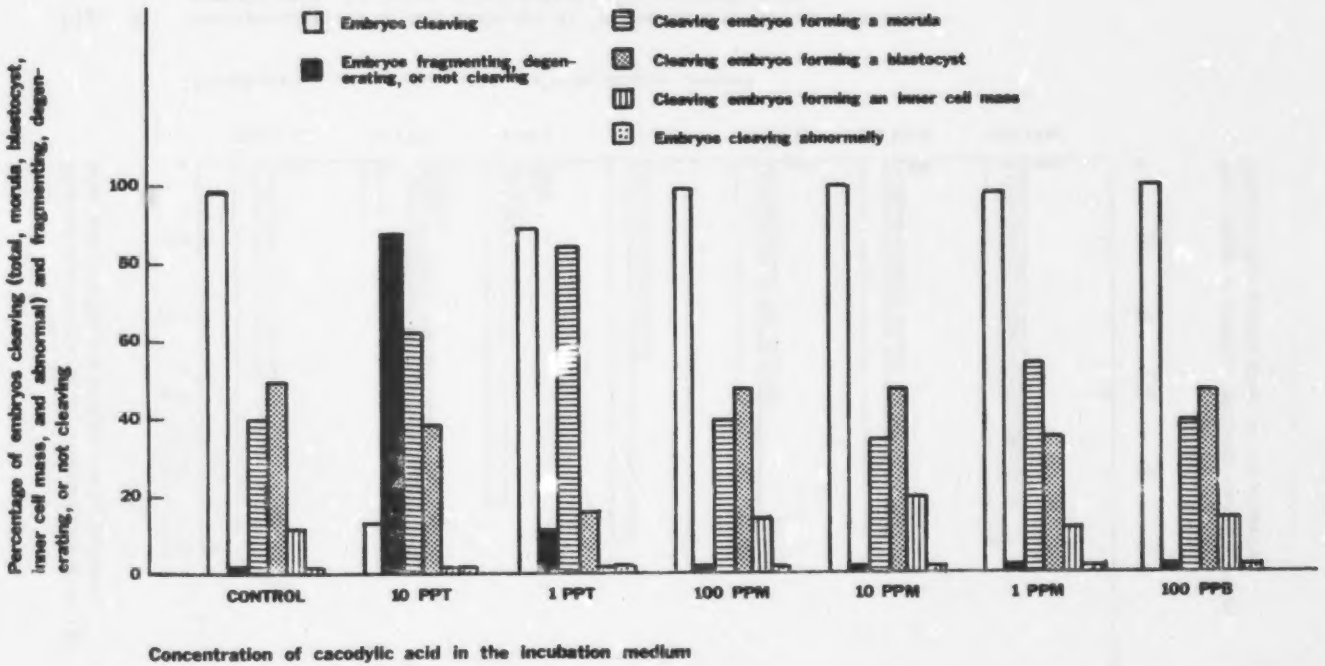

Fig. 4 Developmental response of mouse embryos following 27 hours of culture with cacodylic acid

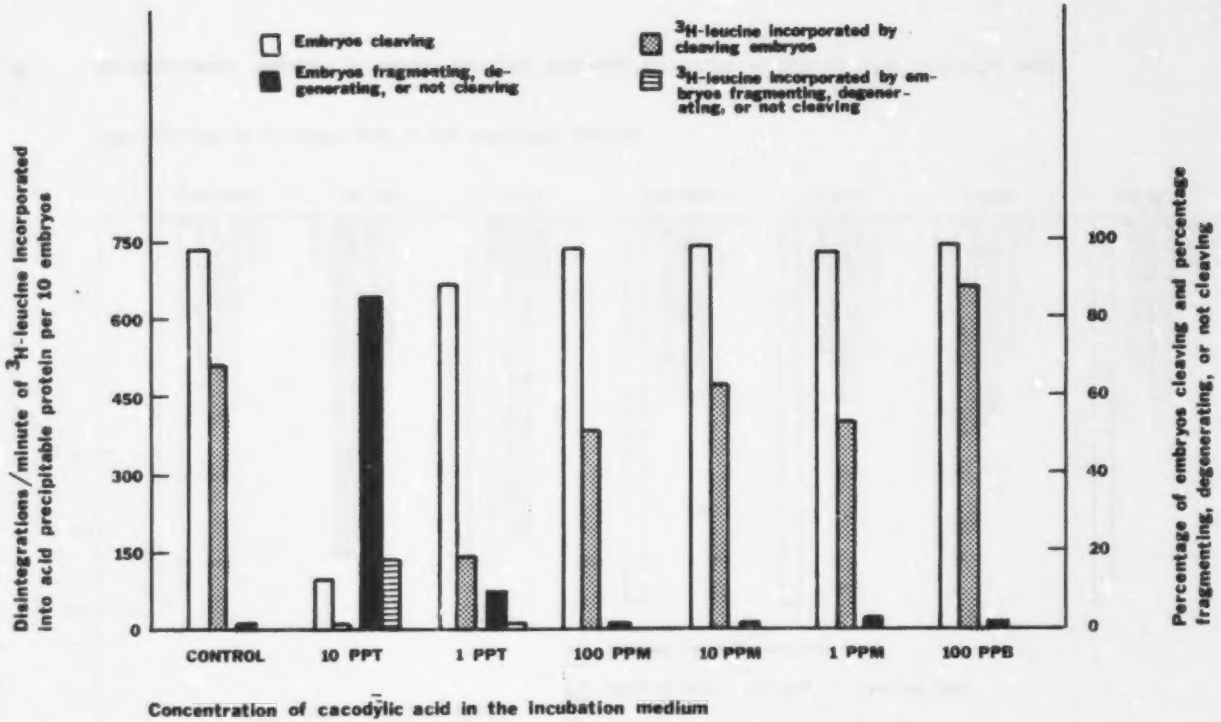

Fig. 5 Incorporation by mouse embryos of ^{3}H-leucine into acid precipitable protein after 24 hours of incubation with cacodylic acid

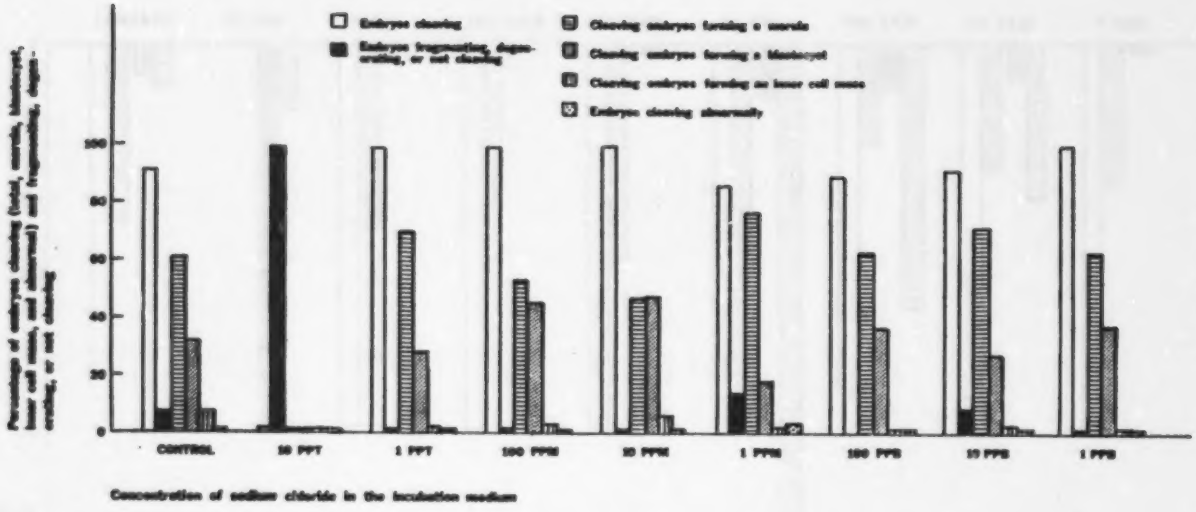

Fig. 6 Developmental response of mouse embryos following 27 hours of culture with sodium chloride

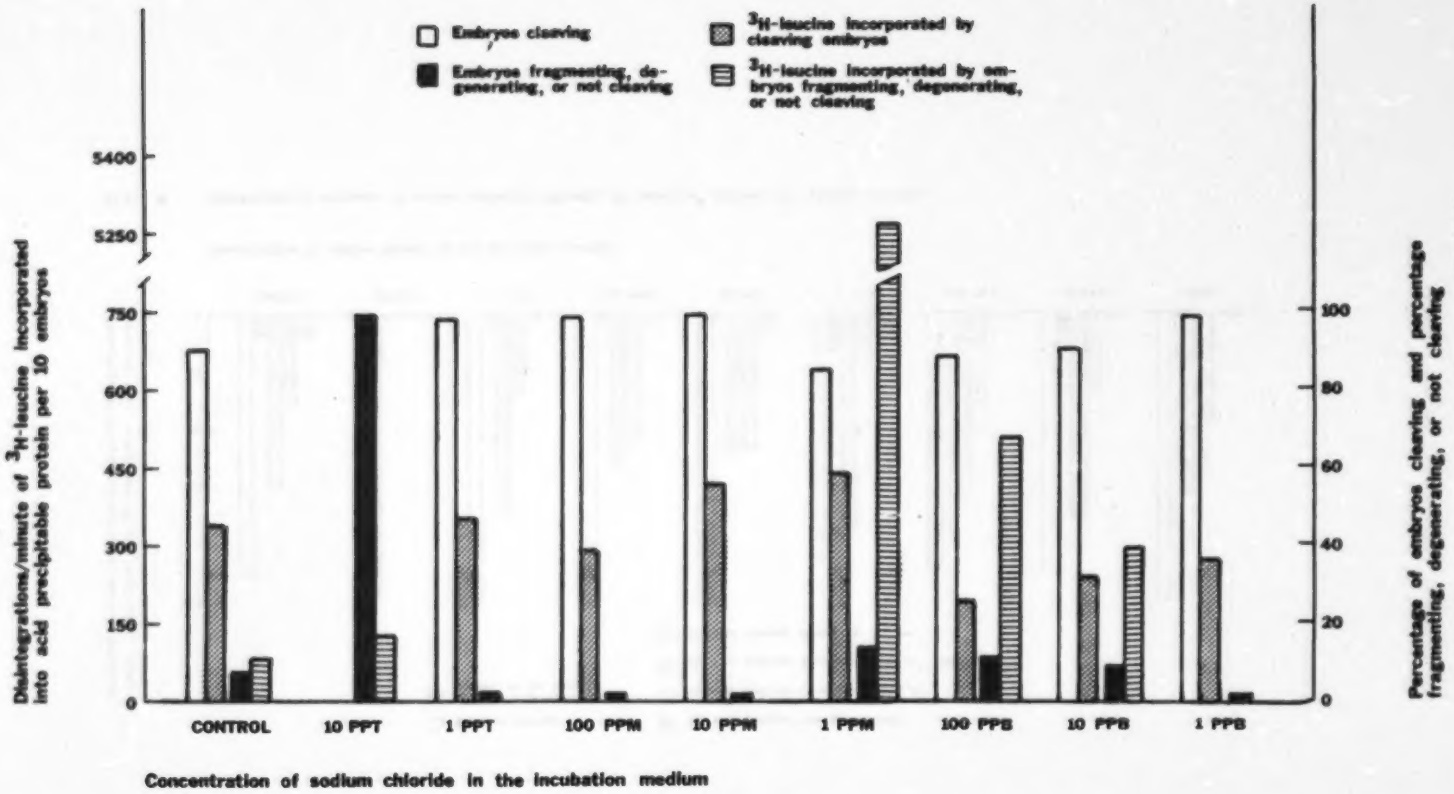

Fig. 7 Incorporation by mouse embryos of ^{3}H-leucine into acid precipitable protein after 24 hours of incubation with sodium chloride

Table 4. Developmental response of mouse embryos cultured in the presence of cacodylic acid

Treatment	No. of incubations	Total No. of embryos	Embryos fragmenting, degenerating, or not cleaving	Embryos cleaving	Stage of development of cleaving embryos			
					ICM	B	M	A
Control	23	221	3 1%	218 99%	23 11%	108 50%	87 40%	0 0%
10 ppt	10	96	83 87%	13 14%	0 0%	5 39%	8 62%	0 0%
1 ppt	9	86	9 11%[c]	77 90%[c]	0 0%	12 16%	65 84%[c]	0 0%[c]
100 ppm	24	230	3 1%	227 99%	31 14%	107 47%	89 39%	0 0%
10 ppm	14	140	0 0%	140 100%	28 20%	66 47%	46 33%	0 0%
1 ppm	14	135	2 2%	133 99%	14 11%	47 35%	72 54%	0 0%
100 ppb	14	131	0 0%	131 100%	18 14%	62 47%	51 39%	0 0%

[c]Significantly different from control ($P < .05$)

Table 5. Metabolic response of mouse embryos cultured in the presence of cacodylic acid

Treatment	Total No. of embryos	Embryos cleaving				Embryos fragmenting, degenerating or not cleaving			
		No. of incubations	No. of embryos	Percent of total	DPM	No. of incubations	No. of embryos	Percent of total	DPM
Control	221	23	218	99	519	2/0[a]	3/0[b]	1	---
10 ppt	96	3/1[a]	13/10[b]	14	0	9	83	86	136
1 ppt	94	10/9[a]	85/84[b]	90	147	1	9	10	0
100 ppm	230	24	227	99	390	3/0[a]	3/0[b]	1	---
10 ppm	140	14	140	100	487	---	---	0	---
1 ppm	135	14	133	98	407	2/0[a]	2/0[b]	2	---
100 ppb	131	14	131	100	671	---	---	0	---

[a]Number of incubations in which embryos responded in a given manner/number of these incubations used for incorporation determinations

[b]Number of embryos having a specific response/number of these embryos used for incorporation determinations

[c]Significantly different from control ($P < .05$)

Table 6. Developmental response of mouse embryos cultured in the presence of sodium chloride

Treatment	No. of incubations	Total No. of embryos	Embryos fragmenting, degenerating, or not cleaving	Embryos cleaving	Stage of development of cleaving embryos			
					ICM	B	M	A
Control	14	134	10 7	124 93%	9 7%	40 32%	75 61%	0 0%
10 ppt	9	83	83 100[c]	0 0%[c]	0 0%	0 0%	0 0%	0 0%
1 ppt	9	91	1 1%[c]	90 99%[c]	2 2%	25 28%	63 70%	0 0%
100 ppm	10	96	1 1%[c]	95 99%[c]	2 2%	43 45%	50 53%	0 0%
10 ppm	7	62	0 0%[c]	62 100%[c]	4 6%	29 47%	29 47%	0 0%
1 ppm	7	65	9 14%[c]	56 86%[c]	1 2%[c]	10 18%[c]	43 77%[c]	2 4%[c]
100 ppb	9	75	8 11%	67 89%	0 0%[c]	25 37%[c]	42 63%[c]	0 0%[c]
10 ppb	9	87	8 9%	79 91%	2 3%[c]	21 27%[c]	56 71%[c]	0 0%[c]
1 ppb	7	62	0 0%[c]	62 100%[c]	0 0%[c]	23 37%[c]	39 63%[c]	0 0%[c]

[c]Significantly different from control ($P < .05$)

Table 7. Metabolic response to mouse embryos cultured in the presence of sodium chloride

Treatment	Total No. of embryos	Embryos cleaving				Embryos fragmenting, degenerating or not cleaving			
		No. of incubations	No. of embryos	Percent of total	DPM	No. of incubations	No. of embryos	Percent of total	DPM
Control	134	13	124	92	346	3/1[a]	10/8[b]	8	85
10 ppt	83	--	---	0	---	9	83	100	126
1 ppt	91	9	90	99	355[c]	1/0[a]	1/0[b]	1	---
100 ppm	96	10	95	99	295[c]	1/0[a]	1/0[b]	1	---
10 ppm	62	7	62	100	423[c]	---	---	0	---
1 ppm	65	6	56	86	431[c]	1	9	14	5265
100 ppb	75	8	67	89	189[c]	1	8	11	506
10 ppb	87	8	79	91	235[c]	1	8	9	290
1 ppb	62	7	62	100	268[c]	---	---	0	---

[a]Number of incubations in which embryos responded in a given manner/number of these incubations used for incorporation determinations

[b]Number of embryos having a specific response/number of these embryos used for incorporation determinations

[c]Significantly different from control ($P < .05$)

Cadmium. A significant effect on the number of embryos cleaving was caused by 100 ppm and 10 ppm (0 and 0 vs. 98% for control). Since all cleaving embryos achieved the morula stage no effect on development was observed. Among the cleaving embryos there was no significant difference in ^{3}H-leucine incorporated into protein (Table 8, Figure 8).

Fluorouracil. All embryos treated with fluorouracil, as well as those which served as controls, were morula at the time of examination. There was no effect of treatment upon cleavage or stage of development (Table 9). In Table 9 a disparity can be observed in the two types of blanks processed. Those blanks taken from control media were much higher than those of treatment media. This may have been caused by an unknown microbial growth in the culture wells during the incubation. This disparity in blanks and the response of embryos in 100 ppb F do not allow for a meaningful interpretation of the data.

Cacodylic Acid and Sodium Chloride. In each of these sets of data, Tables 9 and 10 respectively, the blank is high and the data for the leucine incorporated is erratic. No interpretation is possible from these data. The data on the embryos' ability to cleave is acceptable. It can be observed that either 10 ppt of cacodylic acid or NaCl, and 1 ppt of cacodylic acid inhibits cleavage.

Swine Embryos

From 31 gilts 187 embryos were placed in culture and treated as a control or treated with a dilution of fluorouracil or cadmium. Few of the embryos completed one cleavage when cultured in SOF, and the incorporation data do not reflect valid measurements (Table 12).

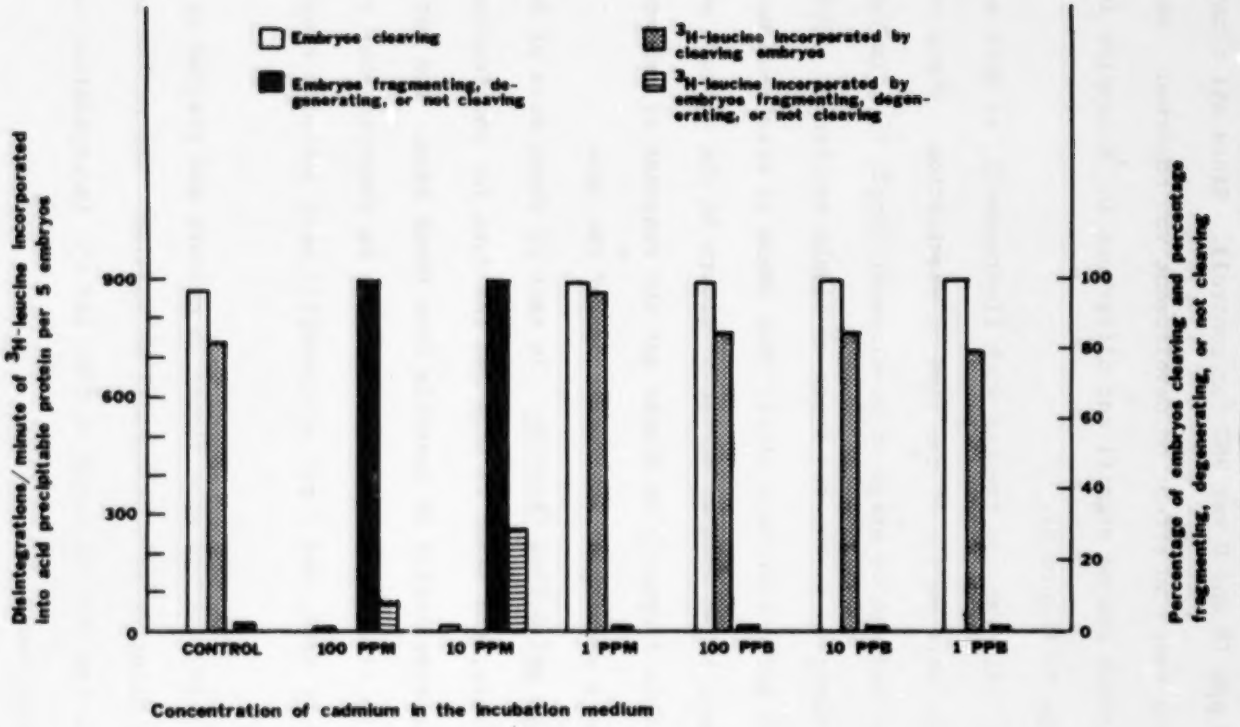

Fig. 8 Incorporation by rabbit embryos of ^{3}H-leucine into acid precipitable protein after 24 hours of incubation with cadmium

Table 8. Developmental and metabolic response of rabbit embryos cultured in the presence of cadmium

Treatment	Total No. of embryos	Embryo cleaving				Embryos fragmenting, degenerating or not cleaving			
		No. of incubations	No. of embryos	Percent of total	DPM	No. of incubations	No. of embryos	Percent of total	DPM
Control	40	8	39	98	757	1/0[a]	1/0[b]	2	---
100 ppm	25	-	--	0[c]	---	5	25	100	74
10 ppm	25	-	--	0[c]	---	5	25	100	263
1 ppm	25	5	25	100	882	-	--	0	---
100 ppb	25	5	25	100	767	-	--	0	---
10 ppb	20	4	20	100	771	-	--	0	---
1 ppb	20	4	20	100	721	-	--	0	---
Blank		2			166				

[a]Number of incubations in which embryos responded in a given manner/number of these incubations used for incorporation determinations.

[b]Number of embryos having a specific response/number of these embryos used for incorporation determinations.

[c]Significantly different from control ($P < .05$)

Table 9. Developmental and metabolic response of rabbit embryos cultured in the presence of fluorouracil

Treatment	Total No. of embryos	Embryo cleaving			
		No. of incubations	No. of embryos	Percent of total	DPM
Control	34	7	34	100	24,662
1 ppt	15	3	15	100	444
100 ppm	30	6	30	100	59
10 ppm	25	5	25	100	772
1 ppm	20	4	20	100	56
100 ppb	20	4	20	100	0
10 ppb	15	3	15	100	82,337
Blank[1]		11			69,059
Blank[2]		3			1,779

[1]Subtracted from control

[2]Subtracted from treatments

Table 10. Developmental and metabolic response of rabbit embryos cultured in the presence of cacodylic acid

Treatment	Total No. of embryos	Embryo cleaving				Embryos fragmenting, degenerating or not cleaving			
		No. of incubations	No. of embryos	Percent of total	DPM	No. of incubations	No. of embryos	Percent of total	DPM
Control	53	11	53	100	27,741	--	--	0	--
10 ppt	29	--	--	0[c]	--	6	29	100[c]	9,401
1 ppt	30	5	25	83[c]	102,730	1	5	17[c]	0
100 ppm	25	5	24	96	27,419	1/0[a]	1/0[b]	4	---
10 ppm	30	6	30	100	21,512	--	--	0	---
1 ppm	25	5	25	100	14,036	--	--	0	---
100 ppb	25	5	25	100	61,406	--	--	0	---
Blank		5			3,832				

[a]Number of incubations in which embryos responded in a given manner/number of these incubations used for incorporation determinations.

[b]Number of embryos having a specific response/number of these embryos used for incorporation determination.

[c]Significantly different from control ($P < .05$).

Table 11. Developmental and metabolic response of rabbit embryos cultured in the presence of sodium chloride

Treatment	Total No. of embryos	Embryo cleaving				Embryos fragmenting, degenerating or not cleaving			
		No. of incubations	No. of embryos	Percent of total	DPM	No. of incubations	No. of embryos	Percent of total	DPM
Control	34	7	33	97	49,596	1/0[a]	1/0[b]	3	---
10 ppt	34	2	6	18[c]	0	6	28	82[c]	417
1 ppt	35	7	30	86	21,963	2	5	14	0
100 ppm	35	7	34	97	0	1/0[a]	1/0[b]	3	---
10 ppm	34	7	33	97	0	1/0[a]	1/0[b]	3	---
1 ppm	30	6	30	100	19,469	---	---	0	---
100 ppb	25	5	22	88	14,375	1	3	12	0
10 ppb	25	5	22	88	25,171	1	3	12	11,075
Blank		16			37,568				

[a]Number of incubations in which embryos responded in a given manner/number of these incubations used for incorporation determinations.

[b]Number of embryos having a specific response/number of these embryos used for incorporation determinations.

[c]Significantly different from control ($P < .05$)

Table 12. Metabolic response of swine embryos cultured in the presence of fluorouracil or cadmium

Treatment	No. of incubations	No. of embryos	DPM of ^{3}H-leucine incorporated/ 5 embryos
Control	16	71	632
100 ppm F	3	11	1206
10 ppm F	5	22	409
1 ppm F	3	14	620
100 ppb F	5	19	1026
Control	7	26	361
10 ppm Cd	3	13	244
1 ppm Cd	3	11	580

DISCUSSION

It is obvious from these observations that a direct relationship exists between the morphology and function of the mammalian embryo and the concentration of certain contaminants which may reach the embryo's environment during its early stages of development. Four different substances are used in the culture medium to demonstrate this relationship. Two of the substances, cadmium and fluorouracil, used in this investigation are known to be teratogenic. A third substance, cacodylic acid, is a substance which is of interest because it comes from a family of compounds known to have teratogenic effects. The fourth substance, sodium chloride, a substance which is required for normal embryo growth; was used as a control substance. It is teratogenic but only at very high concentrations (Nishimura and Mujamata, 1969).

In order to avoid being misled by responses which are limited to only one species these substances were tested in embryos from three different species of mammals; the mouse, the rabbit and the porcine. Much of the preliminary observation and development of technique was done on mouse embryos because of their availability and ease in handling logistic problems. The rabbit was used as representative of that class of species which have a slightly different reproductive pattern in that ovulation is induced rather than spontaneous. The porcine, the third species studied, was used as a representative of a non laboratory species, a species intermediate between the classical laboratory animal and the primate.

These results support the concept that young mammalian embryos _in vitro_ are responsive to a variety of contaminants which can be incorporated in their environment. Different concentrations of the material in the environment influences the type of response elicited in the embryo. The higher concentration results in the

death of the embryo, but a lower concentration of the material may retard embryo development. Still other concentrations of the material will interfere with the function of the embryo as measured by protein synthesis. For example, cadmium appears to be the most toxic of the substances studied. It causes a high rate of death in the embryos subjected to it. On the other hand, fluorouracil results in a negative linear relationship between the concentration of the substance and the incorporation of leucine into protein. These data suggest that substances such as cadmium are effective over a very limited range of concentrations. However, this substance may also provide linear relationships providing sufficient intermediate concentrations were used in the culture media. To demonstrate the sensitivity of the embryo to contaminates further, it is apparent that certain substances in proper concentrations cause a different response among the individual cells of the embryo. For example, a concentration of 100 ppm of fluorouracil will alter the morphology of some of the blastomeres within a given embryo while other blastomeres appear to be normal.

The observations made on embryos from a second species, the rabbit, demonstrate that this system is effective in more than just the mouse. The response of rabbit embryos as measured by cleavage rate is similar to that observed in the mouse embryos when subjected to the same compound. This indicates that the effect is not limited to one species.

Observations made of swine embryos indicate that a prerequisite for this system requires a culture medium which will support embryo growth in vitro. It is obvious from these observations that the precise environmental condition required for normal growth in vitro is different for the three different species. This simply indicates the precise sensitivity of the mammalian embryo to its immediate environmental surroundings. There was no indication from these observations, given

the proper culture medium for normal growth, that swine embryos would not respond similarly to mouse and rabbit embryos when subjected to these compounds. Consequently, these observations suggest that the embryo from any mammalian species, including primate, would respond to pollutants in the manner described in this study providing a culture medium has been developed which supports normal embryo growth *in vitro* for this particular species. Culture media for normal embryo growth are now available for a number of mammalian species including the primate.

The results of this study support the concept that young mammalian embryos can be used to detect the presence of harmful substances in the environment as a preliminary testing system. It would be rapid, inexpensive and efficient. The observations indicate that such a system is feasible and its continued development should be pursued.

SUMMARY

A one year contract was awarded to develop and test the concept that young mammalian embryos could be used to measure the harmful effects of contaminants that may be teratogens.

Embryos from three species of mammals were used; the mouse, rabbit and swine. Four different substances were studied; 5-fluorouracil, cadmium, cacodylic acid and sodium chloride. About 3300 embryos from mice were used in the development of the technique.

In general, the results of the study indicate that young mammalian embryos are responsive to a variety of contaminants that may be found in their environment. Different concentrations of the contaminant will elicit a different kind of response in the embryo. Higher concentrations result in death, but a lower concentration may retard the development of the embryo. Still other concentrations will interfere with the metabolic function of the embryo. Cadmium appears to be the most toxic of the substances studied. It causes the highest rate of embryo death. On the other hand, fluorouracil results in a negative linear relationship between the concentration of the substance and the incorporation of leucine into protein. It appears that some substances are effective over a narrow range while others are effective over a wider range.

Observations from these studies suggest that the contaminants act similarly on mammalian species. However, it is obvious that each species has its own precise environmental requirements for normal growth in vitro.

It is concluded that the procedure as reported is capable of determining the type of effect that a contaminant will have on the development of the mammalian embryo.

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Appendices

Appendix A

BRINSTER'S MEDIUM FOR OVICULTURE ($BMOC_2$)

Compound	mM	Grams/Liter
NaCl[1]	94.88	5.5460
Na Lac*[2]	25.00	2.2530
Na Pyr[7]	0.25	0.0280
KCl[1]	4.78	0.3560
$CaCl_2$[3]	1.71	0.1890
KH_2PO_4[1]	1.19	0.1620
$MgSO_4.7H_2O$[4]	1.19	0.2940
$NaHCO_3$	25.00	2.1060
Penicillin**		1 ml
Streptomycin***		0.2 ml
Bovine serum albumin, fraction V[6]		1.0000
Glucose[1]		1.0000

Solids are added to 1 liter of double glass distilled H_2O and filtered through a Millipore filter (pore size, 0.45μ) into a sterile flask. After filtration, flask openings are covered with sterile gauze, and the flask is placed in a 37° incubator (atmosphere 5% CO_2, 95% air) overnight. Sterile serum bottles are filled with the medium using a sterile Cornwall tubing apparatus under an ultra violet hood. After bottling, pH is checked. Ideal pH after gassing and bottling is 7.4 ± 0.05. $BMOC_2$ is stored under refrigeration for up to 3 months.

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*4.73 ml of 60% sodium lactate can be used per liter instead of neutralized lactic acid. (Sigma, DL-Lactic Acid, 60% Grade V, Sodium Salt).

**Add 10 ml sterile double glass distilled H_2O to 1 million units penicillin; add 1 ml of this solution to 1 liter of culture medium (Squibb, Buffered Potassium Penicillin G, 1 million units) 100 units/ml.

***Add 4 ml sterile double glass distilled H_2O to 1 gm Strep; add 0.2 ml of this solution to 1 liter of culture medium (Lilly, Streptomycin Sulfate U.S.P.) 50 μg/ml.

Sources for chemicals

[1]Fisher for Scientific

[2]Sigma

[3]Allied Chemicals

[4]J. T. Baker Chemical

[5]Matheson, Coleman & Bell

[6]Nutritional Biochemicals

[7]Calbiochem

Appendix B

SYNTHETIC OVIDUCT FLUID (SOF)

Compound	Gram/liter of stock solution (0.154 M, except where noted)	Milliliters of stock solution for 50 ml of culture medium
$NaCl$[1]	9.001	34.96
KCl[1]	11.482	2.325
KH_2PO_4[1]	20.958	0.386
$CaCl_2.2H_2O$[5]	14.702*	0.855
$MgCl_2$	14.665	0.159
$NaHCO_2$[1]	12.938	8.140
Na Lac**[2]	6.469	1.071
Na Pyr[7]	11.100 (0.1M)	0.165
Glucose[1]		dry 13.5 mg
Bovine serum albumin (fraction V) +6		dry 1.6 gm
Penicillin	100 units	
Streptomycin	50 μgm.	
H_2O		0.990

From refrigerated stock solutions and solids, SOF is mixed and filtered through a Millipore filter (pore size, .45) into a sterile flask. Penicillin and streptomycin are added as in $BMOC_2$. After filtration, flask openings are covered with sterile gauze. Medium is gassed overnight in a 37°C incubator with an atmosphere of 5% CO_2 and 95% air. The following morning the medium is put in Sterile serum bottles using a sterile Cornwall tubing apparatus. A bacteriology hood with UV lighting is used for bottling the medium. SOF is refrigerated until needed.

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*$CaCl_2$ = 0.100 M stock

**4.73 ml of 60% sodium lactate can be used per liter instead of neutralized lactic acid.

+ For a flushing solution of SOF 50 mg BSA was added instead of 1.6 gm

Sources of chemicals

[1]Fisher Scientific

[2]Sigma

[3]Allied Chemicals

[4]J. T. Baker Chemical

[5]Matheson, Coleman & Bell

[6]Nutritional Biochemicals

[7]Calbiochem

Appendix C

PROTOCOL FOR THE SUPEROVULATION OF RABBITS

1. Inject 0.4 mg FSH (Armour Baldwin) in methyl cellulose* (1 mg/ml) for 4 days, subcutaneously.
2. On the fourth day of FSH treatment the doe is mated to a fertile male.
3. Breeding is followed by intravenous injection of 2.5 mg of PLH (Armour Baldwin). Diluent is saline (5 mg LH/ml of saline).

*Methyl cellulose (100 centipoise - Fisher). Add 1 mg. methyl cellulose per ml distilled H_2O and put in serum bottles with serum stoppers. Autoclave at 15 lbs., 250^{o}F for 30 min.